The distribution of precursors and biosynthetic enzymes required for Tyrian purple genesis in the hypobranchial gland, gonoduct, and egg masses of *Dicathais orbita* (Gmelin, 1791) (Neogastropoda: Muricidae)

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ABSTRACT

The biosynthetic origin of Tyrian purple in the adult hypobranchial gland and egg masses of the Muricidae is mknown. Histochemistry and mass spectrometry were employed to determine the distribution of biosynthetic components essential for Tyrian purple precursor synthesis within the hypobranchial gland, gonoduct, egg masses, and larvae of *Dicathais orbita*. Histochemical correlations suggest that *de novo* synthesis of the prochromogen, tyrindoxyl sulphate, not only occurs within the hypobranchial gland, but also within the gonoduct, capsule, intraeapsular fluid, and eneapsulated larvae. The coineidence of tyrindoxyl sulphate and arylsulphatase in the capsule and albumen glands, along with the capsule wall and intraeapsular fluid, suggest that the biosynthetie eomponents required for Tyrian purple synthesis are introduced during eapsule formation. Overall it appears that the egg mass natural produets of the Muricidae arise from a maternal souree.

Additional keywords: Bromoperoxidase, arylsulphatase, tyrindoxyl sulphate, capsule, intracapsular fluid, vitellus, natural products

INTRODUCTION

Tyrian purple is an ancient dye of religious and royal significance (Reinhold, 1970) obtained exclusively from hypobranchial gland secretions of muricid mollusks (Cooksey, 2001). Although the east Mediterranean Tyrian purple industry of the 13th Century B.C. once flourished (McGovern and Michel, 1985), traditional dye production has now been all but abandoned (Naegel and Cooksey, 2002). Nevertheless, the historical importance of Tyrian purple has prompted considerable investigation into the chemical composition and formation of this dye.

In 1909, Friedländer elucidated the dominant dye pigment as 6,6'-dibromoindigo (Figure 1, **6**). Much later, Baker and Sutherland (1968) isolated the prochromogen, tyrindoxyl sulphate (Figure 1, **2**) from the Australian murieid, *Dicathais orbita* (Gmelin, 1791). Prochromogen hydrolysis by arylsulphatase (Dubois, 1909; Baker and Sutherland, 1968) and subsequent oxidation and dimerization generates a suite of brominated intermediate dye precursors (Figure 1, **3–5**) (Cooksey, 2001). Of these, tyriverdin (Figure 1, **4**) is photolytieally cleaved to yield the pigment 6,6'-dibromoindigo (McGovern and Michel, 1990; Cooksey, 2001). Depending on proehromogen composition, 6,6'-dibromoindirubin, monobrominated indoles and indirubins, indigo and indirubin may also be formed (Wouters and Verhecken, 1991; Wouters, 1992; Koren, 1995; Cooksey, 2001; Cooksey and Withnall, 2001; Karapanagiotis and De Villemeruil, 2006; Westley and Benkendorff, 2008). Despite the wealth of information available on dye genesis from indoxyl sulphate preeursors, few investigations have focused on the biosynthetic origin of prochromogens and the significance of this biosynthetie pathway.

Secondary metabolite synthesis typically occurs through the modification of primary metabolie pathways. Indoles are believed to arise from the essential amino acid tryptophan (Figure 1, **1**) (Fox, 1983, Verhecken, 1989; Zinderman, 1990). Indeed, storage of tryptophan has been reported within muricid hypobranchial glands where Tyrian purple genesis is known to oceur (Bolognani-Fantin and Ottaviani, 1981; Srilakshmi, 1991; Naegel and Aguilar-Cruz, 2006). Among other enzymatic conversions, tryptophan must then be brominated (Figure 1) to produce the prochromogen tyrindoxyl sulphate (Westley et al., 2006). Bromoperoxidase activity has been detected in hypobranchial extracts of the muricid *Hexaplex trunculus* (Linnaeus, 1758) (Jamun and Coe, 1987), which provides evidence for precursor bromination and hence, *de novo* prochromogen synthesis.

Early observations by Aristotle in ~350 B.C. (Peck, 1970) and Pliny the Elder in 1st century A.D. (Bailey,

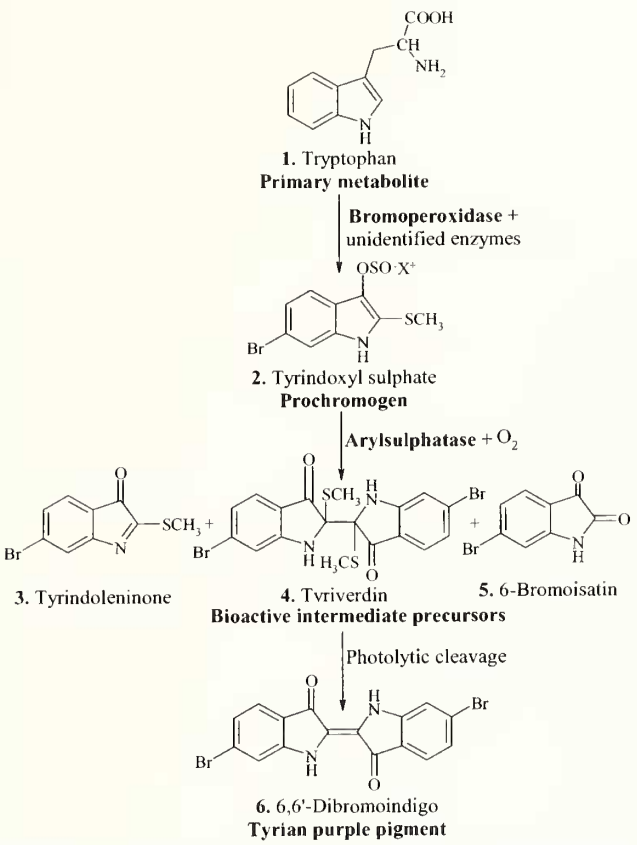

Figure 1. The proposed biosynthetic pathway to Tyrian purple from tryptophan in the muricid *Dicathais orbita* (adapted from Westley et al. 2006).

1929) indicated a link between Tyrian purple genesis and reproduction (Westley et al., 2006; Westley and Benkendorff, 2008). This association was overlooked until Tyrian purple and intermediate precursors were recently isolated from muricid egg masses (Palma et al., 1991; Benkendorff et al., 2000, 2001, 2004). Subsequent observations reported deep red pigmentation in the gonoduct of *Dicathais orbita* (Gmelin, 1791) (Benkendorff et al., 2004) and mass spectroscopic analysis confirmed the presence of Tyrian purple and its precursors (Westley and Benkendorff, 2008). Although these findings imply a fundamental role for these secondary metabolites in the reproduction and encapsulated development of the Muricidae, the capacity for biosynthesis outside the hypobranchial gland remains unknown.

It is currently assumed that the compounds in egg masses arise through maternal investment during capsule formation. However, larvae may possess the capacity to synthesize precursors *de novo*. Natural product biosynthesis has been suggested to commence at an early larval stage in some nudibranch species (Avila, 2006). Non-viable muricid larvae are known to develop purple pigmentation (St. Amant, 1938; Gallardo, 1973; Spight, 1977; Pechenik, 1982; Roller and Stickle, 1988; Naegel, 2004), which implies relevant biosynthetic competence.

This investigation aims to provide new information on the concurrent distribution of the biosynthetic constituents essential for Tyrian purple synthesis in the hypobranchial gland, gonoduct, and egg masses of *Dicathais orbita*. These compounds and enzymes include tryptophan, bromoperoxidase, tyrindoxyl sulphate, and arylsulphatase. Overall, it is hoped these findings will highlight potential sites of prochromogen and Tyrian purple genesis and establish the importance of these secondary metabolites in muricid reproduction and larval development.

MATERIALS AND METHODS

A total of 27 female *D. orbita* specimens and 15 separately spawned egg masses were sampled from the Fleurieu and Eyre Peninsulas of South Australia. The pallial gonoduct and hypobranchial gland of 12 specimens, and the egg capsules and embryos from capsule glands were fresh-frozen cryostat sectioned (15m). Transverse sections were stained with the acid-hydrolysis method for tyrindoxyl sulphate adapted from Baker and Duke (1976), the bromo-phenol red method for bromoperoxidase modified from Krenn et al. (1989) and Wever et al. (1991) (Westley, 2008), and the post-coupling method for arylsulphatase (Rutenburg et al., 1952).

Gonoducts from 12 females, and capsules from 9 egg masses were fixed in 10% neutral-buffered formalin and paraffin embedded. Transverse sections (5m) were stained with the p-DMAB-nitrite method (Adams, 1957) to determine sites of tryptophan storage. Cryostat and paraffin sections were also stained with Haematoxylin and Eosin (Thompson, 1966), Toluidine Blue (Kramer and Windrum, 1954) and Periodic Acid Schiff (McManus, 1946) for morphological and biochemical comparisons.

Tyrindoxyl sulphate distribution was determined by liquid chromatography-mass spectrometry (LC-MS). Hypobranchial, albumen, and capsule glands were excised from three females and capsules sampled from 12 egg masses. Adult tissues and separate capsule constituents (capsule wall, intracapsular fluid and larvae) were extracted in dimethyl formamide (DMF) and analyzed according to Westley and Benkendorff (2008) by high performance-liquid chromatography (Waters Alliance) coupled to a mass spectrometer (MS, Micromass, Quatro micro™). Tyrindoxyl sulphate was identified by registration of expected mass and isotopic clusters in mass spectra (Westley and Benkendorff, 2008).

RESULTS

The distribution of Tyrian purple precursors and biosynthetic enzymes required for natural product synthesis within the female hypobranchial gland, gonoduct, and egg capsule constituents are summarized in Table 1. Tryptophan was detected by positive p-DMAB-nitrite staining (Figures 2–3) within the hypobranchial gland,

Table 1. The distribution of precursors and enzymes required for Tyrian purple synthesis in the female hypobranchial gland, gonoduct and egg masses of *D. orbita*. +, presence; – absence; IF, intracapsular fluid; **NA,** not attainable.

			Gonoduct		Egg mass		
Compound/ enzyme	Technique	Hypobranchial gland	Albumen gland	Capsule gland	Capsule	IF	Larval vitellus
Tryptophan	Histochemistry	+	+	+	+	+	+
Bromoperoxidase	Histochemistry	+	NA	+	+	+	+
Arylsulphatase	Histochemistry	+	+	+	+	–	+
Tyrindoxyl sulphate	Histochemistry	+	–	+	+	–	+
	LC-MS	+	+	+	+	+	+

gonoduct, egg capsule walls, intracapsular fluid and larval vitellus (Table 1). As indicated by bromophenol-red staining (Figures 4–5), bromoperoxidase displayed an identical distribution (Table 1), although the distribution of bromoperoxidase in albumen gland tissue was not acquired due to problematic posterior gonoduct sectioning. Arylsulphatase was localized within all adult (Figures 6–7) and larval tissues (Table 1) examined and the capsule wall (Figure 6), but not the intracapsular fluid (Table 1). Enzyme activity was generally of high activity in the capsule gland (Figure 6) and low activity in the albumen gland, capsules (Figure 6), and larvae. LC-MS revealed the presence of tyrindoxyl sulphate within the hypobranchial, albumen and capsule glands of *D. orbita* specimens, and all capsule constituents including larvae (Table 1). Prochromogen concentration was below the detectable limit by histochemical techniques in the albumen gland and intracapsular fluid (Table 1).

DISCUSSION

Coincidence of tryptophan, bromoperoxidase, and tyrindoxyl sulphate in the hypobranchial gland of *Dicathais orbita* (Table 1) confirms prochromogen synthesis from the primary metabolite, tryptophan. These findings expand on the known occurrence of bromoperoxidase activity in hypobranchial gland homogenates of *Hexaplex trunculus* (Jannun and Coe, 1987) and provide further evidence for the *de novo* synthesis of brominated indoles in the Muricidae. Detection of these biosynthetic constituents within the capsule gland (Table 1) indicates that prochromogen synthesis is also possible within the muricid gonoduct. The capsule gland functions in the deposition of capsule laminae (Fretter, 1941; D'Asaro, 1988) and correlations between capsule gland and capsule biochemistry (Table 1) confirm the introduction of tyrindoxyl sulphate and the biosynthetic components for prochromogen synthesis during capsule formation. The presence of both tyrindoxyl sulphate and arylsulphatase in capsule walls is supported by previous reports of purple pigmentation in capsules of Muricidae (Benkendorff et al., 2004). Overall, these findings provide a means of incorporating natural products into capsules and eliminate the need to transfer precursors from the hypobranchial gland as previously suggested (Westley et al., 2006).

The concurrence of arylsulphatase and tyrindoxyl sulphate within the capsule gland (Table 1) suggests that intermediate precursor and dye genesis also occurs within this gland. This is supported by detection of brominated indoles in *Dicathais orbita* capsule gland extracts by Westley and Benkendorff (2008). Arylsulphatase was also found to coincide with tyrindoxyl sulphate in the albumen gland (Table 1), which highlights this gland as another prospective site for precursor synthesis. However, as tyrindoxyl sulphate was only detectable by mass spectrometry (Table 1), prochromogen concentration must be comparatively low. This is consistent with the intracapsular fluid of *D. orbita* capsules (Table 1), which is thought to originate in the albumen gland in some Muricidae species (D'Asaro, 1988). Low prochromogen concentrations coupled with low arylsulphatase activity may also explain why bioactive intermediates were not previously reported in autolyzed albumen tissues (Westley and Benkendorff, 2008). Overall, the limited biosynthetic capacity of the albumen gland suggests it is unlikely to contribute significant concentrations of brominated indoles to *D. orbita* egg masses.

In comparison to the intracapsular fluid, larval vitellus was found to contain all the biosynthetic components required for Tyrian purple genesis (Table 1). This is consistent with previous reports of intermediate precursors and Tyrian purple in muricid egg capsule extracts (Palma et al., 1991; Benkendorff et al., 2000, 2001). Muricid embryos are largely composed of nutritive vitellus (Roller and Stickle, 1988; Naegel, 2004). These yolk granules are synthesized by ovarian follicle cells and oocytes (Martel et al., 1986; Amor et al., 2004), and consumed over the course of development (González and Gallardo, 1999). As tryptophan must be derived from the diet (Crawford, 1989; Bentley, 1990; Hermann et al., 1992), it is likely that the ovary contributes tryptophan to yolk granules during vitellogenesis. In the case of bromoperoxidase, arylsulphatase and possibly tyrindoxyl sulphate, it is unclear whether these originate from follicle cells or the oocyte.

The findings of this investigation strongly indicate that bioactive intermediate precursors in the egg masses of *D. orbita* are synthesized within the capsule wall, larval vitellus and, to a lesser extent, the intracapsular fluid, from biosynthetic components of maternal origin. As the caenogastropod pallial gonoduct evolved from an

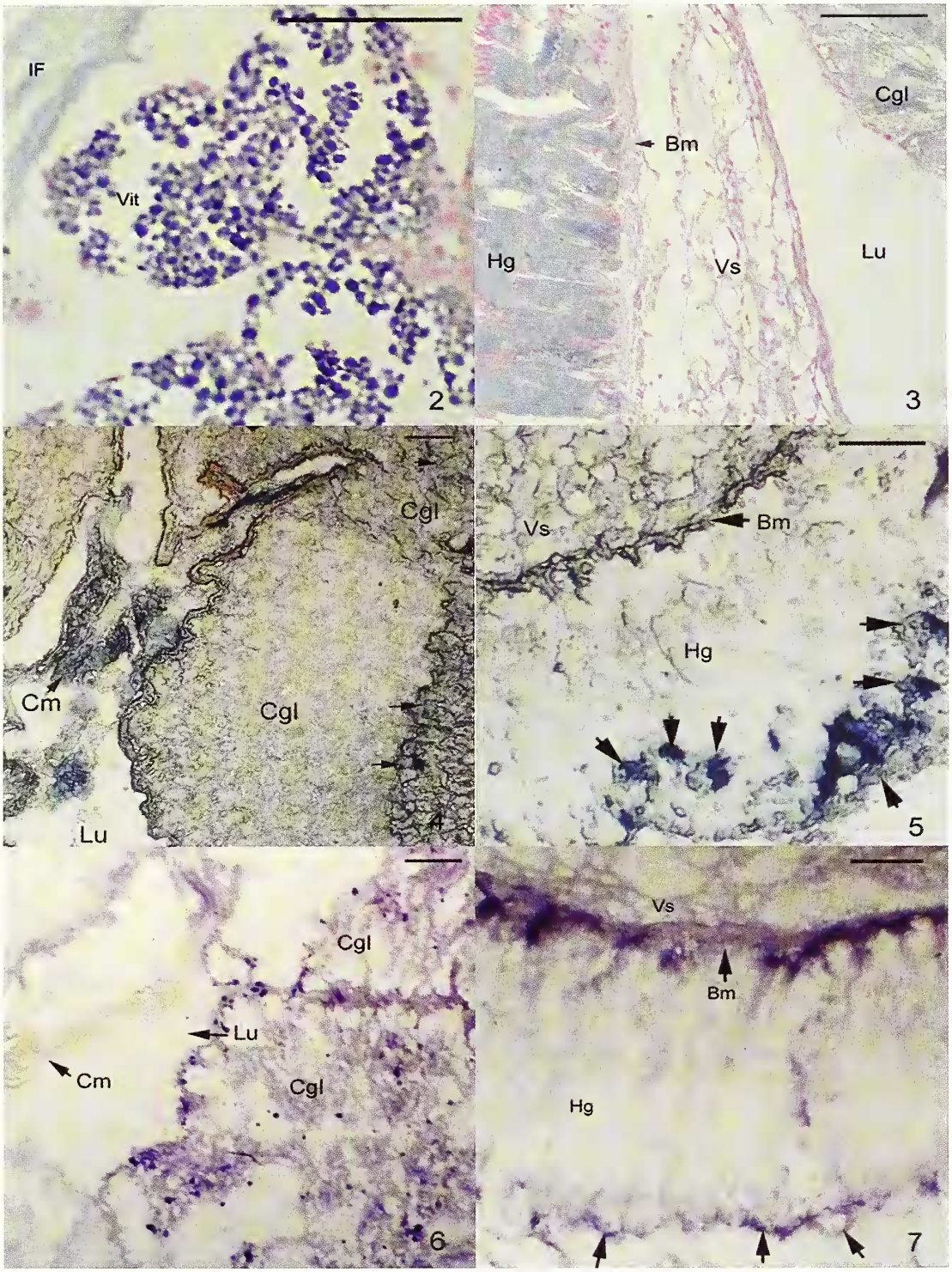

Figure 2–7. *Dicathais orbita.* Transverse histological sections. **2.** Encapsulated larvae, showing tryptophan distribution evidenced by blue p-DMAB-nitrite staining within the vitellus (**Vit**) and intracapsular fluid (**IF**). **3.** Tryptophan distribution evidenced by blue p-DMAB-nitrite staining within the capsule (**Cgl**) and hypobranchial gland (**Hg**). **4.** Capsule gland containing a partially formed egg capsule. Bromoperoxidase activity (**arrows**) indicated by bromophenol-blue staining of capsule material (**Cm**). **5.** Hypobranchial gland, showing bromoperoxidase activity (**arrows**) evidenced by bromophenol-blue staining. **6.** Capsule gland, showing arylsulphatase activity (**arrows**) displayed by red (= low levels) staining of capsule material. **7.** Hypobranchial gland, showing arylsulphatase activity (**arrows**) displayed by purple (= high levels) staining. Abbreviations: **Bm**, basement membrane; **Cgl**, capsule gland; **Cm**, capsule material; **Hg**, hypobranchial gland; **IF**, intracapsular fluid; **Lu**, lumen; **Vit**, larval vitellus; **Vs**, vascular sinus. Scale bars = 100 µm.

ancestral right hypobranchial gland (Fretter et al., 1998), it appears that the capacity for Tyrian purple synthesis has been retained in various reproductive glands of the Muricidae over the course of evolution. The presence of Tyrian purple precursors (Benkendorff et al., 2001, 2004) in the egg masses of species from the monophyletic subfamilies Rapaninae, Muricinae, Ocenebrinae, and Ergalataxinae (Claremont et al. 2008) suggests this phenomenon is widespread in the Muricidae. However, the absence of Tyrian purple precursors from some Ocenebrinae species (Benkendorff et al., 2001, 2004) indicates that gonoduct biosynthesis of hypobranchial gland metabolites may be clade-specific. Nevertheless, this chemotaxonomic divide coupled with male Tyrian purple genesis (Elsner and Spanier, 1985; Verhecken, 1989; Michel et al., 1992; Benkendorff et al., 2004; Westley and Benkendorff, 2008), indicates that maternal provisioning to support larval development is not the sole function of these natural products.

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